ROLE OF RND EFFLUX PUMPS AND BIOFILM IN CONFERRING ANTIMICROBIAL RESISTANCE (AMR)

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

SYNOPSIS OF THE THESIS

BY

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(REGISTRATION NO. 20220021188)

UNDER THE GUIDANCE OF

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Synopsis of the Thesis to be submitted in Partial Fulfilment of the Requirements for the Award of the Degree of Doctor of Philosophy

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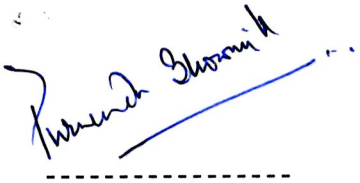

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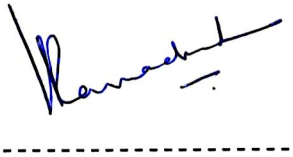

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A. Review Of Literature

A.1 Introduction

Antimicrobial resistance (AMR) is a global health challenge that could jeopardise the effectiveness and desired outcome of many of the currently used antimicrobial treatments. A major hurdle of such immense proportions would consequently lead to failure in the treatment of even simple infections caused by different drug-resistant microbes including bacteria, viruses, parasites, and fungi. The spread and impact of AMR has been recognized as a significant threat to the general public, and the healthcare sector, that could eventually adversely impact the overall welfare and economic development of any society.

The World Health Organization (WHO) has classified the ESKAPE pathogens (representing *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species)* under "priority" category, highlighting a clear need to urgently develop new antibiotics against these to combat AMR[1,2]. ESKAPE pathogens cause a significant proportion of hospital-acquired infections including pneumonia, sepsis, urinary tract infections, and surgical site infections and are most often associated with high rates of multi-drug resistance[3–6]. Members of ESKAPE pathogens exploit multiple strategies to equip themselves to ward off the inhibitory effect of antibiotics thereby developing resistance even against the most potent antibiotics [2,7]. These modes of acquiring resistance include one or a combination of factors, including their ability to acquire resistance-conferring genes, mutation of target genes leading to modification in binding sites and affinity to the drug, and production of enzymes that modify or degrade the drug, while others can alter their cell walls or membrane permeability to prevent drug entry. Apart from all the aforementioned strategies, microbes possess in their armamentarium, efflux pumps employed often, by Gram-negative bacteria that operate by the expulsion of drugs, limiting their concentrations intracellularly[8–11].

A.2 Efflux pumps in AMR

Efflux pumps are membrane transporters that can extrude a wide array of substrates, including toxic metabolites, salts, inducer molecules and many more substrates out of bacterial cells[12–14]. Apart from these, efflux pumps can expel multiple classes of antimicrobial agents, thereby aiding the bacteria to escape their

lethal action. Efflux pumps through this strategy reduce the effective concentration of the drug inside the cell, leading to insufficient levels thereby allowing the bacteria to survive[15]. These pumps are thus instrumental in contributing to drug resistance and at times also indirectly influencing physiological processes adopted for an alternate mode of growth e.g., biofilms, persisters, single cell variants etc. Thus the occurrence of AMR in the clinic could be due to the combined effect of the activities of efflux pumps in conjunction with other mechanisms [16].

Bacterial systems exhibit a diverse array of efflux pumps, which are broadly classified into five major families, namely, the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, the multi-drug and toxic compound extrusion (MATE) family, the major facilitator superfamily (MFS), the small multi-drug resistance (SMR) family, and the resistance/nodulation/division (RND) superfamily. The activity of an efflux pump depends on the different types of energy sources each system uses: ABC transporters are fuelled by ATP hydrolysis; MFS, RND, and SMR use the proton-motive force, and MATE transporters consist of Na^+/H^+ drug antiport systems. Except for the RND superfamily, which is found predominantly in Gram-negative bacteria, efflux systems of the other four families: MFS, ABC, SMR and MATE, are widely distributed in both Gram-positive and Gram-negative bacteria[13,17–19]. Depending on the specific classes they belong to, efflux pumps are either single-component transporters like the SMR and MFS family of efflux pumps or multiple-component systems containing not only an inner membrane transporter but also an outer membrane channel and a periplasmic adaptor protein, such as the RND type efflux pumps[17,20].

A.2.1 RND Family of Efflux pumps

Owing to their wide range of distribution across the ESKAPE pathogens, a broad range of substrate specificities, and their tripartite composition, which allows the direct extrusion of various drugs from cytosol or periplasmic space to the outside of bacterial cells, their contribution to AMR is very well recognized and thus has gained significant attention [21]. Some of the well-reported members in different Gram-negative bacteria are the AcrB in *Escherichia coli*[22–24], MexB in *Pseudomonas aeruginosa*[25,26] and AdeB in *Acinetobacter baumannii*[27,28]. Another member of the efflux pump family, which is frequently associated with AMR, is OqxB, primarily studied in *Klebsiella pneumoniae*[29]. In addition to this native host, OqxB has also been reported to play a decisive role in AMR caused by other Gram-negative bacteria

like *E. coli*, *Enterobacter cloacae* and *Salmonella spp.*, owing to its rapid mobilization through plasmids, insertion element and horizontal gene transfer[30,31]. One of the key features of OqxB is its ability to expel diverse antibiotics including nitrofurantoin, β-lactams, aminoglycosides, quinolones, tigecycline, and colistin[29,32–34]. Such findings warrant a detailed understanding of its role and function and hence, towards this goal, the current study covering the structural-functional analysis and study of its contribution to multi-drug efflux, was embarked upon.

It is well understood that for any drug discovery program, the use of protein crystallography offers meaningful insights into the mechanism of inhibition that brings about interference with the target protein function[35]. Moreover, structure-function analysis of any protein helps to elucidate the substrate/inhibitor binding and its subsequent impact on complex biological activities. Such evaluations are thus, often routinely supported with various modelling and biochemical studies to decipher the overall architecture and functional aspects of efflux pumps. Among the widely distributed members of the RND efflux pumps in Gram-negative organisms, efflux pumps like AcrB in *Escherichia coli*[36,37], MexB in *Pseudomonas aeruginosa*[38–40] and AdeB in *Acinetobacter baumannii*[28,41], are well reported in the literature and characterized in detail. In contrast, our current understanding of the structural insights of the OqxB efflux pump in *K. pneumoniae* is still limited and needs to be explored further, in order to address the threat posed by OqxB-dependent efflux liability. To discern the key features and role of mainly OqxB along with some preliminary understanding of various other RND efflux pumps, the approaches used included heterologous gene overexpression, construction of knockdown strains, combined with structural studies involving protein crystallography, *in silico* modelling etc. The objectives of the doctoral work were framed accordingly.

A.3 Biofilms and AMR

Many bacterial species are known to resort to alternate forms of growth characteristics to tide over nutrient deprivation and harsh environmental conditions. Biofilms, one such lifestyle adaptations, are specialized well defined, three-dimensional (3D) structures which enable bacteria to thrive under stressful conditions. These are protected and hard-to-permeate self-encapsulated bacterial aggregates, that are usually anchored to biotic or abiotic substrates. Such transformations in lifestyle confer growth-related advantages and refractoriness to stressful conditions, immune responses and

more importantly equipped to evade antibiotic action. Biofilms pose a significant threat to the healthcare sector, food industries and also cause biofouling. Thus biofilms have become the primary reasons behind hard to treat chronic infections and also lead to revenue losses in other industrial sectors [42,43]. Furthermore, efflux pumps which are known to throw out antibiotics to escape their lethal action, have also been reported to be pivotal in extruding toxic metabolites. The significance of efflux pumps in biofilm formation can be emphasized further with the fact that many anti-biofilm treatments are often combined with efflux pump inhibitors, resulting in both disruption of biofilms and a decline in the overall resistance[44,45].

Formation of biofilms is a concerted event which mainly involves the contribution of non-essential genes encoding multiple regulatory factors like FlhDC, CsgD, RpoS etc., and structural components like curli, fimbriae, PGA, LPS etc., which together form the overall assembly of this complex structure[46]. A wide array of metabolites like c-di-GMP, c-di-AMP, cGMP, cAMP, (p)ppGpp act as secondary messengers and coordinate with different components and events of bacterial pathogenesis and biofilm formation[47]. For optimal "cell-to-cell communication" among the bacterial population, composed either of mono-microbial origin or as poly-microbial communities, key components like quorum sensing molecules such as AI-2, indole, AHL etc., in conjunction with major participation of efflux pumps are considered crucial and hence, they contribute significantly in biofilm development as well[48]. The vast interconnected network of these critical players contributes to the overall process of biofilm formation, which is driven by the sequential and differential gene expression profiles of multiple factors and components. These events, in turn, facilitate the transition of the motile planktonic cells to a non-motile adherent biofilm form[49,50]. The latter are often implicated in recurrent, chronic, poly-microbial hard to treat infections affecting patients at a global level. Therefore, an in-depth understanding of these underlying interlinked processes behind the building of the complex biofilm architecture would aid the identification of novel targets in biofilm formation and delineate the cause for increased resistance.

Modelling studies based on flux balance analysis (FBA), Boolean logical networks and stochastic principles, apart from molecular genetic approaches have been utilized to assess biofilm formation. Further advancements have led to the development of agent-based and 3D models using partial differential equations[51–54]. Although outputs from these earlier studies added significant value to our current understanding

of biofilms, none of them could successfully capture the interplay of the events and the cross-talks of various components to give an overall holistic understanding of the signalling pathways. To explain and unravel the latter in greater depth, an ODE-based *in silico* platform was constructed to simulate biofilm formation and resistance development. *E. coli* was chosen as the model organism towards this objective.

B. Research Objectives.

Considering the complex nature and the role of multiple players contributing to the phenomenon of AMR, the current work was initiated with the following objectives:

B.1 To study the contribution of RND efflux pump components from different Gram-negative organisms (AcrB in *E. coli*, MexB in *P. aeruginosa* and OqxB in *K. pneumoniae*) in conferring antimicrobial resistance (AMR): a heterologous overexpression-based complementation approach.

B.2 To study the structure-function relationship of *Klebsiella pneumoniae* RND efflux pump OqxB and its role in the efflux of fluoroquinolones.

B.3 Identification of key interacting residues in OqxB and studying their contributions in conferring efflux-mediated resistance against fluoroquinolones.

B.4 Development of a dynamic validated *in silico* model, simulating the functionally inter-connected networks of key components in *E. coli* biofilms.

C. Methodology and Key Findings

C.1 To study the contribution of RND efflux pump components from different Gram-negative organisms (AcrB in *E. coli*, MexB in *P. aeruginosa* and OqxB in *K. pneumoniae*) in conferring antimicrobial resistance (AMR) via the use of a heterologous gene overexpression based-complementation approach.

C.1.1 Cloning and overexpression of AcrB/AcrA-AcrB, MexB/MexA-MexB and OqxB/OqxA-OqxB

To study the effect of different RND efflux pump components in the significant expulsion of common antibiotics, viz., AcrB from *E. coli*, MexB from *Pseudomonas*

aeruginosa and OqxB from *K. pneumoniae*. While the pumps AcrB (*E. coli*) and MexB (*P. aeruginosa*) share 70% sequence homology, the sequence homology with OqxB (*K. pneumoniae*) was < 41%. Since OqxB can move to other bacterial systems via horizontal gene transfer, we selected these components to check for their abilities to form a functional efflux pump complex in *E. coli*, upon heterologous expression. For this, the respective gene(s) were cloned as single or double complementation overexpression systems under a promoter and vector backbone of pTrc99a and pET21a, to ensure their expression in the host. The clones were confirmed by restriction digestion and Sanger sequencing (**Figure 1A and 1B**). The plasmids were transformed in *E. coli* BW25113 and *E. coli* C43(DE3) (wild type and efflux compromised *ΔacrB* single KO and *ΔacrA*- *ΔacrB* double KO) to verify their abilities to form a functional complex and optimally efflux some of the known antibiotics.

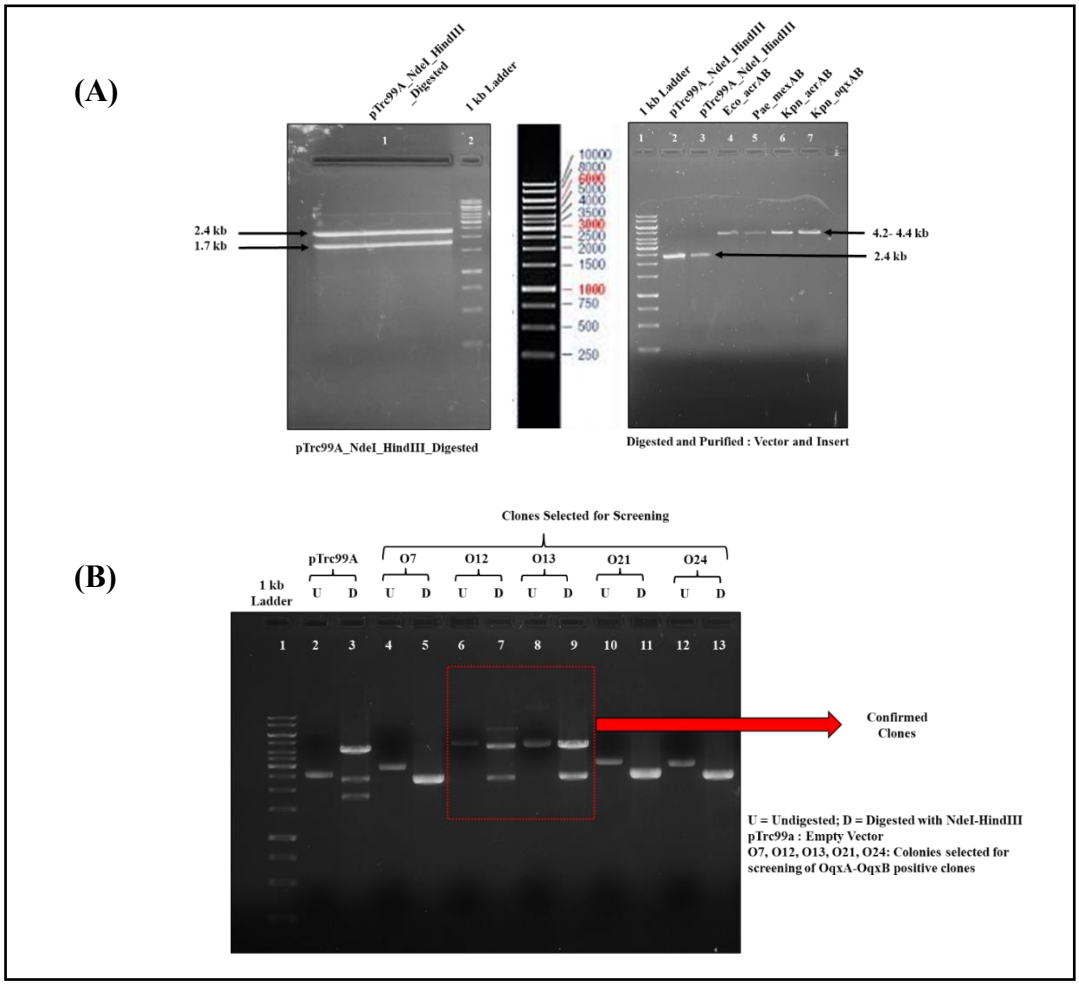


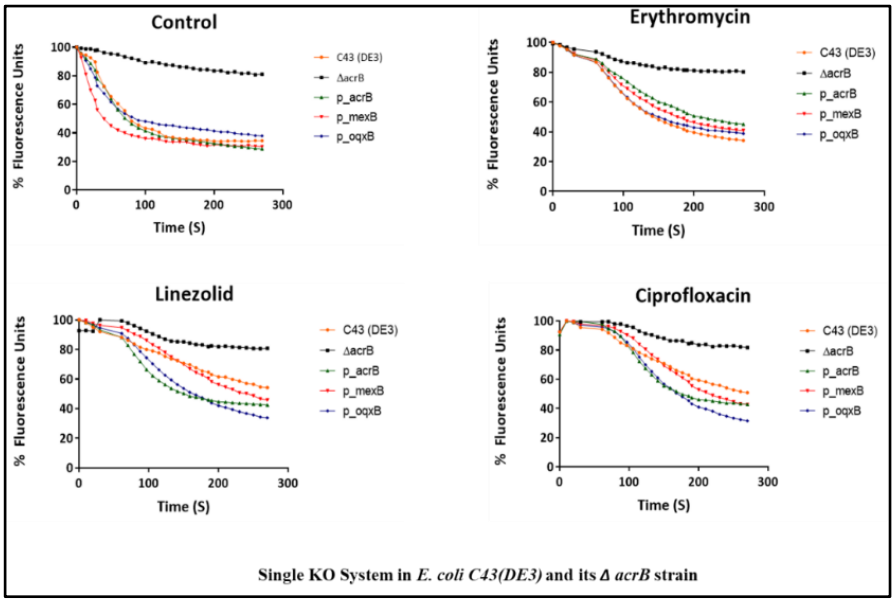
Figure 1. Cloning of the RND efflux pumps homologues from different Gram-negative organisms. (A) Extraction of pTrc99a Vector backbone and PCR products of the gene inserts of Eco_acrA-AcrB, Pae_mexA-mexB, Kpn_acrA-acrB and

Kpn_oqxA-oqxB, amplified from the genomic DNA of the hosts namely *E. coli BW25113* (Eco), *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 13883 respectively (B) Confirmation of the positive clones for Kpn_oqxA-oqxB from the selected colonies, by restriction digestion with HindIII and NdeI. The selected clones were further confirmed for the presence of the gene of interest by Sanger sequencing. A similar approach was followed for the screening of Eco_acrA-acrB and Pae_mexA-mexB positive clones (data not shown).

C.1.2 Efflux Complementation by AcrB/AcrA-AcrB, MexB/MexA-MexB and OqxB/OqxA-OqxB overexpression systems.

Efflux monitoring assays with lipophilic membrane dyes, like Nile Red, are frequently utilized as gold standard assays for assessing the extent of drug extrusion by efflux pumps[55]. This assay is based on the competitive binding of the dye Nile Red and the drug molecules with the efflux pumps, followed by their release into the extra-cellular milieu. The ability of the efflux pump components from three key Gram-negative organisms to form a heterogenous tri-partite pump complex and extrude antibiotics was assessed by the Nile Red efflux assays. The results of these assays indicated that strains overexpressing different (heterologous) pump components were found to effectively extrude Nile red, indicating the assembly of a functional tri-partite system in efflux-deficient *E. coli* (**Figure 2**).

(A)



(B)

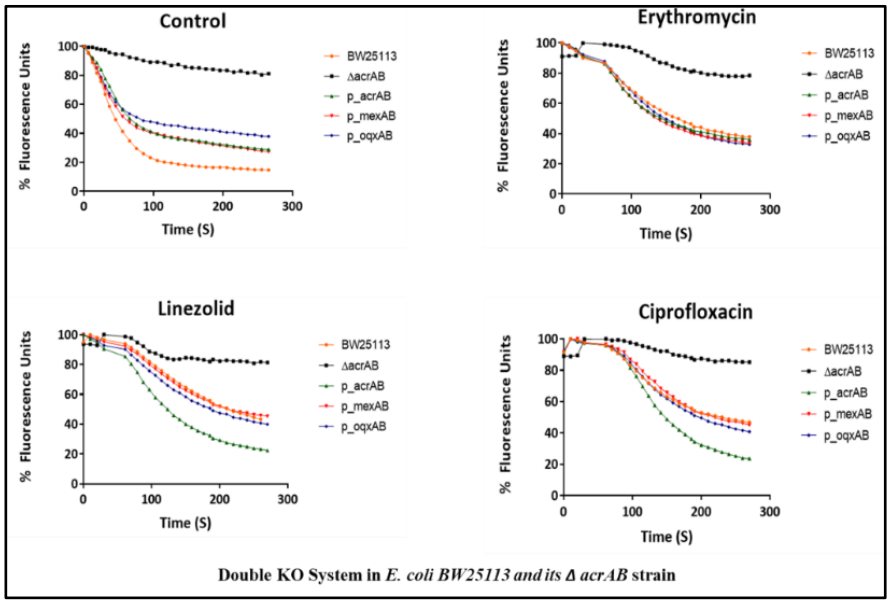


Figure 2. Efflux of Nile Red in the wildtype and efflux deficient ΔacrB/ΔacrAB strains and their complementation. Inhibition of Nile Red efflux by competitor antibiotics with high efflux liability in (A) *E. coli C43(DE3)* (WT) and its single knockout background of ΔacrB, complemented with single overexpression systems of native AcrB or heterologously expressed MexB and OqxB (B) *E. coli BW25113* (WT) and its double knockout background of *ΔacrAB*, complemented with double overexpression systems of native AcrAB or heterologously expressed MexAB and OqxAB.

The functionality of all the overexpressing heterologous systems was further tested by determining the minimum inhibitory concentrations (MICs) in liquid broth media, as per the CLSI guidelines[56], for antibiotics including linezolid (LZD), novobiocin (NOVO), erythromycin (ERY), which are known to have high efflux liabilities in Gram-negative bacteria. The results of the MIC experiments further corroborated the outputs from the Nile Red assays reported earlier. The absence of the native efflux pump component in wildtype systems in either C43(DE3) *ΔacrB* or BW25113 *ΔacrAB* background resulted in potent MICs for all the tested antibiotics by 8-16-fold, thus confirming its role in the extrusion of these antibiotics. However, upon complementation with the efflux pump components, MexB/MexA-MexB, and OqxB/OqxA-OqxB in similar background, the MIC values were restored with an

increased shift in MICs by 8-16 folds and in some cases, back to the wild type levels (**Table 1A and 1B**). These outcomes indicate that the non-native or heterologous overexpressed efflux pump components not only can form a functional pump complex but are also capable of compensating for the loss of the native *E. coli* pump component by mediating the efflux of the tested antibiotics effectively. Additionally, inhibition of AcrB and the other non-native pump systems by efflux pump inhibitors (EPIs) like phenylalanine-arginine-β-naphthylamide (PaβN), improved the activity of all the antibiotics, and the MICs were 10 to 20-fold more potent, indicating that the mechanism of action of the functional tri-partite efflux pump components of heterologous origin is similar to that of the native one. Similar results were observed when some of the antibiotics were evaluated at different concentrations on solid agar plates (**Figure 3**).

A. Single Overexpression Complementation Assays in *E. coli BW25113 ΔacrB* background

	BW25113		ΔacrB	ΔacrB+pTrc99a		ΔacrB+AcrB_OE		ΔacrB+MexB_OE		ΔacrB+OqxB_OE	
PAβN	-	+	-	+	-	+	-	+	-	+	-
LZD	400	25	12.5	25	12.5	400	6.25	400	12.5	400	12.5
NOV	> 400	50	25	50	6.25	400	25	> 400	25	400	6.25
ERY	160	1.25	10	10	0.3125	40	0.625	80	0.3125	80	0.625
MOX	0.05	0.025	0.0125	0.025	0.0125	0.025	0.00625	0.0125	0.0125	0.025	0.0125
RIF	5	< 0.07	5	5	< 0.004	5	< 0.07	5	0.15	5	0.15

B. Double Overexpression Complementation Assays in *E. coli BW25113 ΔacrAB* background

	BW25113		ΔacrAB	ΔacrAB+pTrc99		ΔacrAB+AcrAB_OE		ΔacrAB+MexAB_OE		ΔacrAB+OqxAB_OE	
PAβN	-	+	-	-	+	-	+	-	+	-	+
LZD	200	25	25	25	12.5	100	12.5	100	25	100	25
NOV	400	50	50	25	3.125	400	25	400	25	100	6.25
ERY	160	1.25	40	20	10	160	5	80	0.625	80	1.25
MOX	0.025	0.0125	0.0125	0.025	0.0125	0.025	0.012.5	0.05	0.025	0.1	0.0125
RIF	5	2.5	5	2.5	0.4	5	< 0.08	5	< 0.08	5	< 0.08

Table 1(A) and 1(B). Minimum Inhibitory Concentrations (MIC; values in µg/ml) MIC values of the antibiotics tested for their efflux liabilities and extrusion in Single and double overexpression systems; **PABN:** Phenylalanine-Arginine-beta-Naphthylamide (PAβN); 25µg/ml; **LZD**: Linezolid; **NOV**: Novobiocin; **ERY**: Erythromycin; **MOX**: Moxifloxacin; **RIF**: Rifampicin

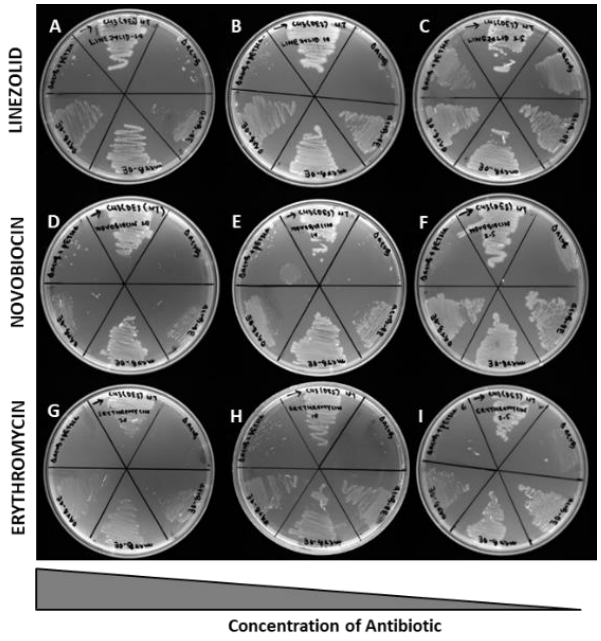


Figure 3. Determination of MIC on agar plates to check for complementation by overexpressing acrB, mexB and oqxB in *E. coli C43(DE3)* Δ*acrB* background. Different concentrations of compounds like linezolid (LZD): Plates A-C; novobiocin (NOV): Plates D-F and erythromycin (ERY): Plates G-I, were tested to check the sensitivity of the strains expressing different pumps in *E. coli C43(DE3)* Δ*acrB*.

C.2. To study the structure-function relationship of *Klebsiella pneumoniae* RND efflux pump OqxB and its role in the efflux of fluoroquinolones.

OqxB is an RND (Resistance-Nodulation-Division) efflux pump that has emerged as a critical factor contributing to the antibiotic resistance seen in *Klebsiella pneumoniae* and its dissemination via horizontal gene transfer to other Gram-negative bacterial pathogens including *Escherichia coli, Enterobacter cloacae* and *Salmonella spp*. In this study, we describe the crystal structure of OqxB with n-dodecyl-β-D-maltoside (DDM) molecules bound in its substrate-binding pocket, at 1.85 Å resolution. We utilized this structure in computational studies to predict the key amino acids contributing to the efflux of fluoroquinolones by OqxB. The amino acid residues were distinct from related efflux pump components, AcrB and MexB. Moreover, minimum inhibitory concentration (MIC) determinations with clinical isolates of *E. coli* provided further evidence that the predicted structural features are indeed involved in ciprofloxacin efflux.

C.2.1 Crystal Structure of OqxB Efflux pump from *Klebsiella pneumoniae*

OqxB from *K. pneumoniae* was purified and its crystal structure was solved at 1.85 Å resolution. (PDB ID: 7CZ9). The asymmetric unit contained six OqxB monomers/protomers and each monomer was composed of 1040 residues. The overall fold of the trimer was similar to other published HAE1 sub-family of RND class of efflux pumps such as AcrB and MexB. Each OqxB monomer contained 12 trans-membrane (TM) helices (TM1 to TM12). The large periplasmic domain of the OqxB protomer that emerged from the inner membrane was connected through two extracellular loops that link TM1 with TM2 and TM7 with TM8 (**Fig. 4A**). The six individual protomers (**Fig. 4B**) were structurally similar with main chain atoms RMSD of less than 0.8 Å.

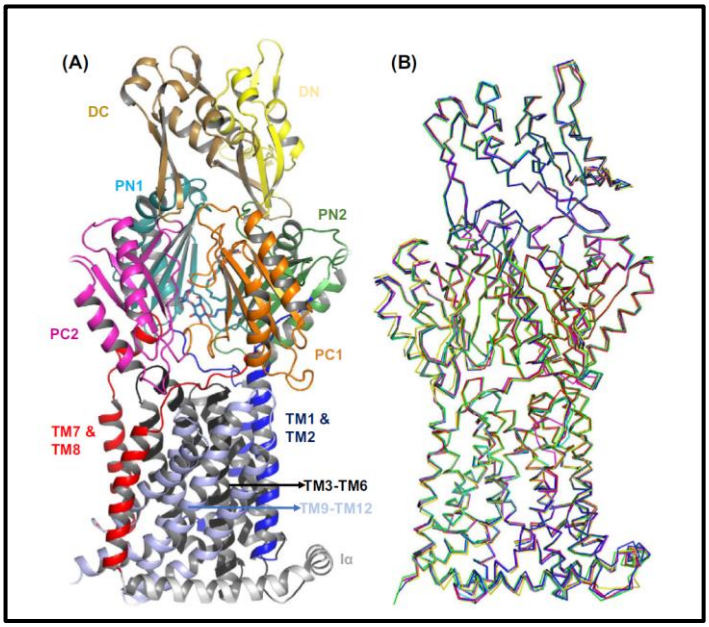


Figure 4. OqxB structural features. The OqxB structure single protomer (A) with important sub-domains is highlighted with varied colours and labelled accordingly. The porter sub-domains PC1, PC2, PN1, and PN2 are highlighted with orange, magenta, cyan, and green colour cartoon, respectively. The six OqxB protomers (B) superimposed to demonstrate the identical nature of individual protomers.

Although OqxB has a comparatively low sequence identity with other RND efflux pumps, our crystal structure illustrated similarities in their architecture. Several published RND efflux pump structures have revealed that they exist as an asymmetric trimer such that each protomer has a different conformation; access/loose,

binding/tight, and extrusion/open. The efflux pump's asymmetric trimer provided the basis for its functional rotation mechanism for the drug/substrate extrusion process. In contrast, the OqxB structure is symmetric, wherein all three protomers are in a binding/tight protomer state with two DDM molecules bound to each protomer. Moreover, sequence and structural comparisons offered important evidence to speculate that OqxB can also exist as an asymmetric trimer.

C.2.2 Substrate-binding pocket comparison with other RND pumps

The OqxB protomer substrate-binding pocket is located deep inside the periplasmic cleft, between the β-sheets of PN2 and PC1sub-domains. Comparison of the substrate binding pocket of OqxB, with AcrB and MexB, revealed some of its salient features. Although the substrate binding pocket in AcrB and MexB is primarily rich in hydrophobic residues like phenylalanine (F136, F178, F610, F628) and a few hydrophilic residues like serine (S48, S155) and asparagine (Q125), it has aspartate (D274) a charged amino acid. In contrast, the substrate binding pocket of OqxB exhibits a high propensity for charged residues and is also rich in both hydrophobic residues like R48, R157, R774, E50, D87 and E184 and F180, F618, F636, L138, L280 and L621 respectively (**Figure 5A and 5B**).

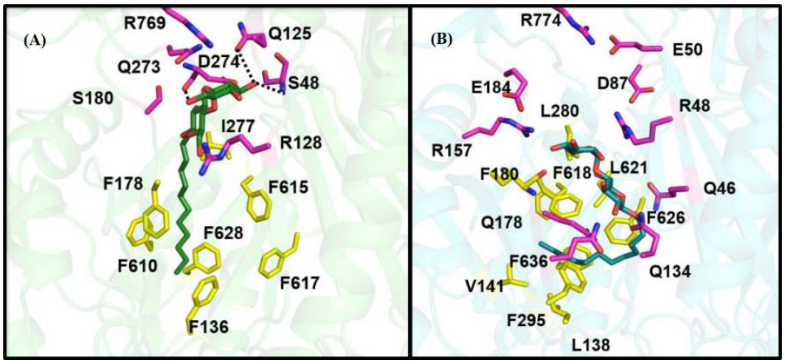


Figure 5. Binding mode differences of DDM and LMNG molecules. Experimentally determined DDM binding mode with MexB (A) and OqxB (B) structures compared. MexB binding protomer (PDB ID:3W9I) superimposed on OqxB protomer to capture the differences. Hydrophobic residues are shown as yellow sticks whereas hydrophilic residues are depicted as magenta sticks and labelled accordingly. MexB-bound DDM molecule is shown as green, OqxB-bound DDM molecule is shown as cyan, and LMNG molecule as pale magenta colour sticks.

Significant differences were also found in the gate loops (g-loops) that are part of the PC1 sub-domain and residues close to the entrance tunnel in OqxB, AcrB, and MexB. Several structural studies with mutants demonstrate that the g-loop is important to allow the substrate molecules into the substrate-binding pocket and facilitate the efflux process. While the g-loop in AcrB and MexB is primarily composed of glycine and phenylalanine residues, the composition of this loop in OqxB is somewhat unique and different. In addition to some of the conserved glycine residues (G620: equivalent to G614 of AcrB and MexB) on OqxB loop, there are two proline residues (P619 and P630) and small hydrophobic residues such as L621 and A623, which replaces the two phenylalanine residues (F615 and F617) present on g-loop of both AcrB and MexB. This is further compensated by F626 which swings back towards the substrate-binding pocket, facilitated by the extended loop, as well as the presence of prolines on both sides of the loop thereby contributing to the phenylalanine cage architecture of the OqxB (**Figures 6 and 7**).

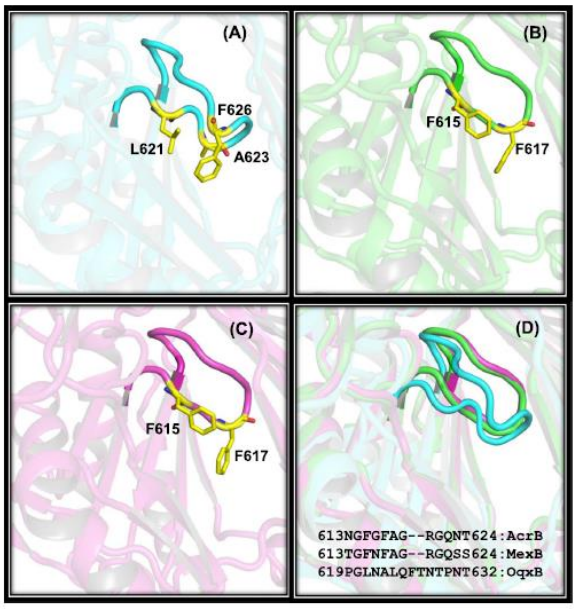


Figure 6. The gate loop (g-loop) variations. Structural and sequence level differences of the g-loop are shown by highlighted loop structure and important residues of OqxB (A), AcrB (B), and MexB (C). Important hydrophobic residues which are crucial for substrate binding were highlighted as yellow color sticks and labelled. All three structures were superimposed to emphasize the g-loop orientational differences of OqxB compared with the other two structures (D). Sequences of g-loop aligned to highlight the extra residues of OqxB.

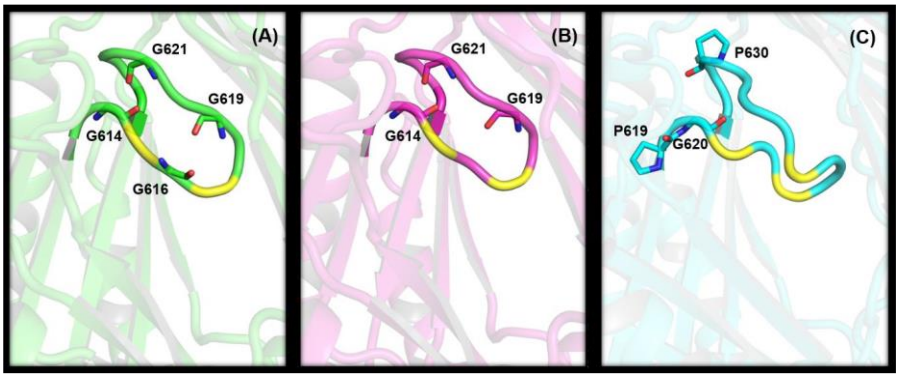


Figure 7: Residual composition of g-loop. The glycine or proline residues present on AcrB (A), MexB (B) and OqxB (C) of the g-loop are highlighted. These residues are shown as sticks and residue numbers are labelled. Important hydrophobic residues which are crucial for substrate binding are also highlighted with yellow colour.

C.2.3 Role of OqxB efflux pumps in fluoroquinolone resistance

To ascertain the binding mode of OqxB with the three fluoroquinolones (ciprofloxacin, levofloxacin, and moxifloxacin), a computational molecular docking approach followed by MD simulations was utilized. Residues around the DDM1 molecule were considered as binding pocket residues to generate the grid for docking simulations. All three fluoroquinolones possessed a consistent binding mode at the substrate-binding pocket. The fluoroquinolone carbonyl group interacts with the S182 main chain amino group, and the quinolone aromatic ring makes pi-cation interaction with the R157 guanidino side chain. A positively charged group of the fluoroquinolone molecules entered the hydrophilic pocket near the exit funnel and had hydrogen bond interactions with the E50 side chain. The OqxB hydrophilic pocket constitutes three negatively charged (E50, D87, and E184), as well as three positively charged residues (R48, R157, and R774). This highly charged OqxB hydrophilic pocket is another remarkable difference that is not observed in any other RND efflux pumps (**Figure 8**). In addition, the intra-molecular interactions between the important charge residues pairs (E50:R774 and R157:E184) showed stable interactions consistent with the crystal structure and were crucial for the binding and subsequent efflux of fluoroquinolones.

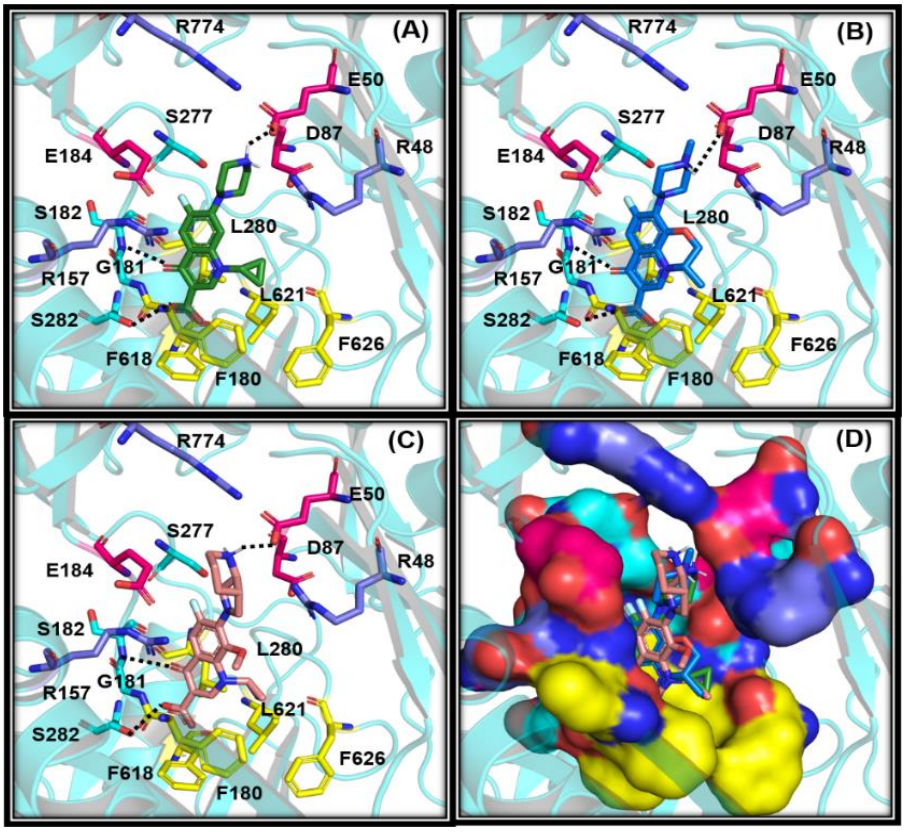


Figure 8. Predicted binding modes of fluoroquinolones with OqxB. Molecular docking predicted binding orientations of ciprofloxacin (A), levofloxacin (B), and moxifloxacin (C). Hydrophobic, positively charged, and negatively charged residues are depicted as yellow, blue, and magenta sticks, respectively. Other important substrate-binding pocket residues are shown as cyan colour sticks. Intermolecular hydrogen bond interactions are highlighted with broken lines. All the predicted docking modes are superposed to illustrate similar interaction patterns of fluoroquinolones with OqxB substrate-binding pocket (D).

The role of OqxB in the binding and subsequent efflux of fluoroquinolones was further established by complementation and MIC experiments, in which the heterologous over-expression of OqxB / OqxA-OqxB lead to a significant rise in the MIC values by 8-16 folds, thus confirming its role in the extrusion of fluoroquinolones like ciprofloxacin, moxifloxacin as well as other common antibiotics with high efflux liability like linezolid and novobiocin (**Table 2 and Table 3**). A corresponding over-expression of the native *E. coli* AcrB did not result in any significant shift in the MICs for fluoroquinolones, further indicating the specificity of OqxB towards fluoroquinolones.

Compounds	*E. coli C43(DE3)*	*ΔacrB*	*ΔacrB* + pET21a	*ΔacrB* + *acrB*	*ΔacrB* + *oqxB*
LZD	**200**	**12.5**	**12.5**	**100**	**200**
NOV	**400**	**12.5**	**12.5**	**400**	**100**
CIP	**0.00625**	**0.00625**	**0.00625**	**0.00625**	**0.1**
LEV	**0.00625**	**0.0125**	**0.00625**	**0.0125**	**0.1**
MOX	**0.00625**	**0.0125**	**0.0125**	**0.025**	**0.2**
RIF	**5**	**5**	**5**	**5**	**5**

Table 2. MIC values with acrB and oqxB complementation in *E. coli* **C43(DE3)-*ΔacrB*.** MIC values in µg/mL; *E. coli C43(DE3)* or its *ΔacrB* deletion strain used for MIC; pET21a, + *acrB or* + oqx*B*: complementation with plasmid carrying the respective genes; LZD: linezolid; **NOV**: novobiocin; **CIP**: ciprofloxacin; **LEV**: levofloxacin; **MOX**: moxifloxacin; **RIF**: rifampicin.

Compounds	*E. coli* BW25113 (WT)	*ΔacrAB*	WT + Plasmid pTrc99a	WT + *acrAB*	WT + *oqxAB*
LZD	**400**	**12.5**	**400**	**200**	**> 400**
NOV	**200**	**6.25**	**400**	**200**	**> 400**
CIP	**0.00625**	**0.00625**	**0.00625**	**0.00625**	**0.05**
LEV	**0.025**	**0.00625**	**0.0125**	**0.0125**	**0.2**
MOX	**0.025**	**0.0125**	**0.0125**	**0.025**	**>0.4**
RIF	**10**	**5**	**5**	**10**	**10**

Table 3. MIC values with acrB and oqxB complementation in *E. coli* **BW25113 (WT).** MIC values in µg/mL; *E. coli* BW25113 (WT: wildtype) or its Δ*acrAB* deletion strain used for MIC; pTrc99a, + *acrAB,* +oqxA*B*: complementation with a plasmid carrying the respective genes; **LZD**: linezolid; **NOV**: novobiocin; **CIP**: ciprofloxacin; **LEV**: levofloxacin; **MOX**: moxifloxacin; **RIF**: rifampicin.

C.3 Identification of key interacting residues in OqxB and studying their contributions in conferring efflux-mediated resistance against fluoroquinolones.

In the current study, the detailed characterization of the structural architecture of the OqxB efflux pump and the identification of unique amino acid residues within its substrate binding pocket has been reported. The results from this study led to the identification of the crucial interactions of olaquindox and various fluoroquinolones like ciprofloxacin, norfloxacin, levofloxacin and moxifloxacin at the substrate binding pocket (**Figure 9**). Further, the importance of the positively charged residues (R48, R157 and R774) and the negatively charged residues (E50, D87 and E184) were also established.

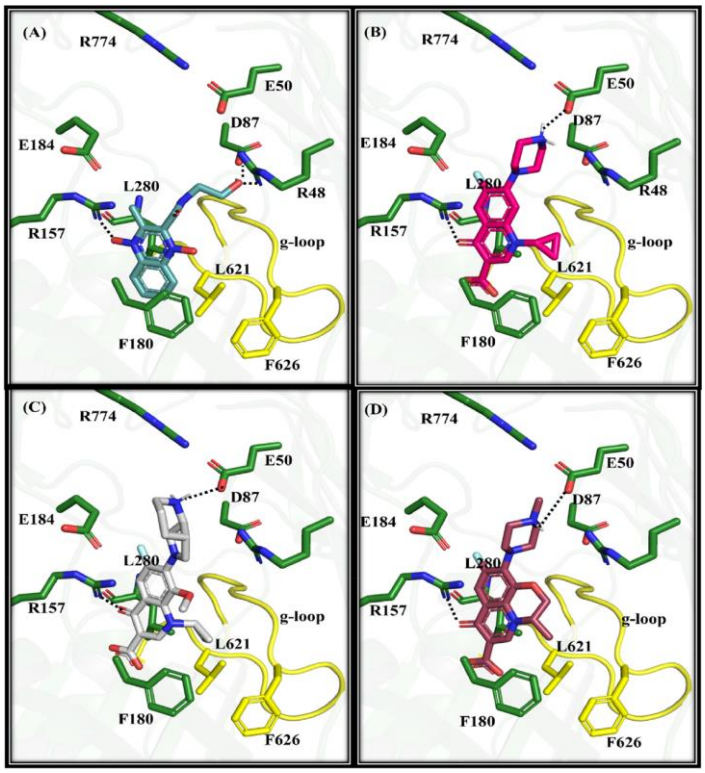


Figure 9. Molecular Docking for the predicted binding modes of olaquindox (A) ciprofloxacin (B), moxifloxacin (C), and levofloxacin (D) at the substrate binding pocket or phenylalanine cage of OqxB. Olaquindox is depicted as dark cyan whereas all three fluoroquinolones: ciprofloxacin (magenta), moxifloxacin (grey) and levofloxacin (brown) sticks. Important interacting residues as well as ion pairs shown as dark green sticks and hydrogen bond interactions between protein and ligand are shown as broken lines. The g-loop (gate loop) which is key for substrate entry into the phenylalanine cage highlighted in yellow colour and the two key hydrophobic residues are shown as sticks.

Contrary to the other RND efflux pumps, the effective binding of the substrate at the OqxB substrate binding pocket involved the combined contributions of the hydrophobic pocket with residues like F180A, L280, L621and F626, as well as the charged residues of OqxB. Their critical roles in the binding and efflux of fluoroquinolones based on the predictions by docking studies and MD simulations were ascertained and validated using site-directed mutagenesis and MIC determination. This work demonstrated that the unique structural components of the OqxB efflux pumps comprising of features such as the combination of aromatic ring systems with charge residues, along with zwitterionic/hydrophilic substitutions could be the influencing factor, which in turn dictates its ability to extrude certain classes of antibiotics.

Molecular docking studies revealed some residues at the substrate binding pocket of OqxB, which are crucial in fluoroquinolone binding and their subsequent extrusion. To validate the roles of these residues, crucial hydrophobic residues (F180, L280, L621 and F626) and the charged residues (R48, E50, D87, E184 and R774) were selected for site-directed mutagenesis and MICs determined. For these studies, the heterologous system comprising of *K. pneumoniae* wildtype or mutated OqxB was constitutively overexpressed in *E. coli* BW25113. Since most of the studies using clinical isolates of *Klebsiella spp.*, *E. coli* and *Salmonella spp*. that exhibit fluoroquinolone resistance are usually found to be associated with the presence or transmission of plasmid-mediated quinolone resistance (PMQR) of OqxB via horizontal gene transfer, *E. coli* wildtype was chosen as the host strain for our studies, instead of the efflux pump deleted double knockout strains (*ΔacrAB*). Such an approach would mimic the possible scenario encountered among the clinical isolates in the human host environment. For the complementation studies, host cells transformed with pTrc99a served as the control system. The complementation studies with wildtype and mutated *Klebsiella pnuemoniae* OqxB, in *E. coli BW25113* system exhibited an altered resistance pattern exhibiting a high degree of specificity towards fluoroquinolones. The overexpression of wildtype OqxA-OqxB in *E. coli* significantly enhanced the MIC values, resulting in an enhanced shift of 8-16 folds (in comparison to wild-type *E. coli*). However, the substitution of the hydrophobic residues with alanine using site-directed mutagenesis, resulted in a complete reversion of the fluoroquinolone extrusion ability of the OqxB efflux pump, as evidenced by the restored MIC values, similar to that seen with wild-type *E. coli BW25113* (**Figure 10**). In contrast, the overexpression of the

native AcrA-AcrB in *E. coli* did not show any considerable shift in the MIC values for antibiotics like olaquindox, ciprofloxacin, norfloxacin, moxifloxacin, and levofloxacin, further confirming the specificity of OqxB in extrusion of fluoroquinolones.

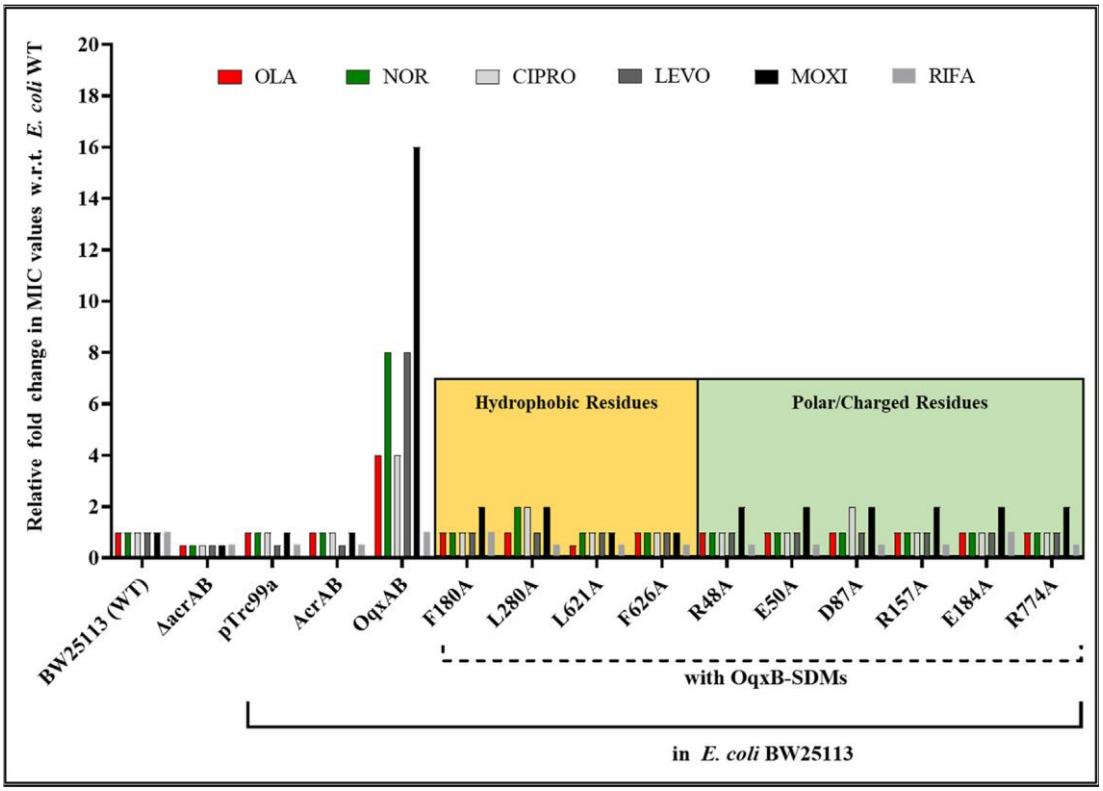


Figure 10. Relative Fold-change in MIC values. Relative fold change for the MIC values of olaquindox and different fluoroquinolones in *E. coli* BW25113 (WT) complemented with either *K. pneumoniae* OqxA-OqxB or OqxA-OqxB (with SDMs) overexpression systems; Rifampicin was used as a negative control. pTrc99a, pAcrAB, pOqxAB, pOqxA-pOqxB(SDMs): complementation with a plasmid carrying the respective genes or the gene with indicated mutations. OLA: olaquindox; NOR: norfloxacin; CIPRO: ciprofloxacin; LEVO: levofloxacin; MOXI: moxifloxacin; RIFA: rifampicin.

C.4 Development of a dynamic and validated *in silico* model, simulating the functionally inter-connected networks of key components in *E. coli* biofilms.

In the previous studies, we evaluated the role of efflux pumps and their contribution to AMR. Another strategy used by different bacterial species which leads to enhanced resistance is their switch to biofilms, an alternate lifestyle to escape and survive in harsh environmental conditions. The alternate living style helps to bring to the fore activities of key target proteins that contribute to improved survival, enhanced virulence, and development of resistance during the biofilm formation. The objective of this study was thus to have insights behind the transformation from a free-living planktonic to a sessile, self-encased multicellular 3-dimensional structure. Such a study could lead to the development of small molecule inhibitors that disrupt biofilms and also to understand the key components responsible for increased resistance.

C.4.1 Development of validated *in silico m*odel

The process of biofilm formation is driven mostly by non-essential genes. Therefore, published literature on around 300 genes associated with different pathways involved in biofilm formation was curated and static maps were created. These maps were suitably interconnected with their respective metabolites or components and integrated as a single dynamic platform using ordinary differential equations (ODE). The precise interplay of genetic networks and signalling pathways that regulate the transitory switching of bacterial growth patterns in response to environmental changes and the resultant multicomponent synthesis of the extracellular matrix was appropriately modelled to build a dynamic platform. Using the R and Python environment, time-dependent changes in the concentration of various components were simulated. The model was validated by predicting the impact of key gene knockouts (KOs) on biofilm formation and comparing the findings with that reported in the literature. For example, the role of extracellular DNA (eDNA) on biofilm formation was validated in the model by simulating a graded knockdown (KD) study, to check its impact on the overall biofilm formation. (**Figure 11**). Multiple such iterations and alignment were carried out with all the components to complete the construction of a validated model.

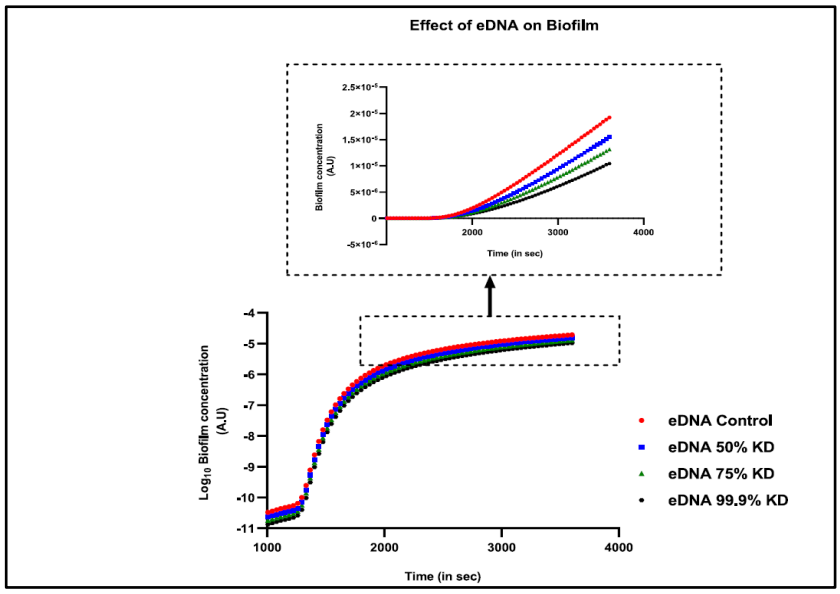


Figure 11. Role of eDNA in biofilm formation. eDNA is an integral component of the biofilm matrix and the same has been validated in the model by running simulations with a graded reduction (50%, 75%, and 99.9% KD) in the overall levels of eDNA with respect to control levels. As evident, a graded reduction in the levels of eDNA in the system resulted in a decline in biofilm formation. The plots represented here are the simulation output from the *in silico* platform, as a result of graded KD of eDNA levels in the model (inset: the effect of eDNA graded perturbation on biofilm formation represented in a linear scale).

Control: WT or control simulations; 50% KD: 50% knockdown; 75% KD: 75%knockdown; and 99.9% KD: 99.9% knockdown (equivalent to KO), done with respect to control levels.

A logarithmic scale has been used for a better representation of the data.

A.U.: arbitrary unit; used for the representation of the relative levels of the model output.

C.4.2 Identification of novel targets and validation of their role in biofilm formation.

Following the development of a validated model, the study was extended to identify key targets whose disruption would negatively impact biofilm formation as well as to understand the link to efflux pumps for their roles in biofilm and resistance development. This included members of the two-component signal transduction systems (like EvgAS), global regulatory proteins (like RpoS, YdeO), structural proteins

(like curli, fimbriae), matrix components (like PGA), secondary metabolites (like ppGpp, c-di-GMP), quorum sensing molecules (like AI-2, indole), along with the multi-factorial roles of efflux pumps in quorum sensing (LsrA) and extrusion of toxic metabolites and drugs (AcrDF and MdtEF). From the predictive simulations of the model, three novel target genes namely *miaA, ydeO*, and *ygiV* were found to influence biofilm formation. Each of these targets has a distinct regulatory network, through which they exert their effect on the overall process of biofilm formation and enhanced multi-drug resistance (**Figure 12**).

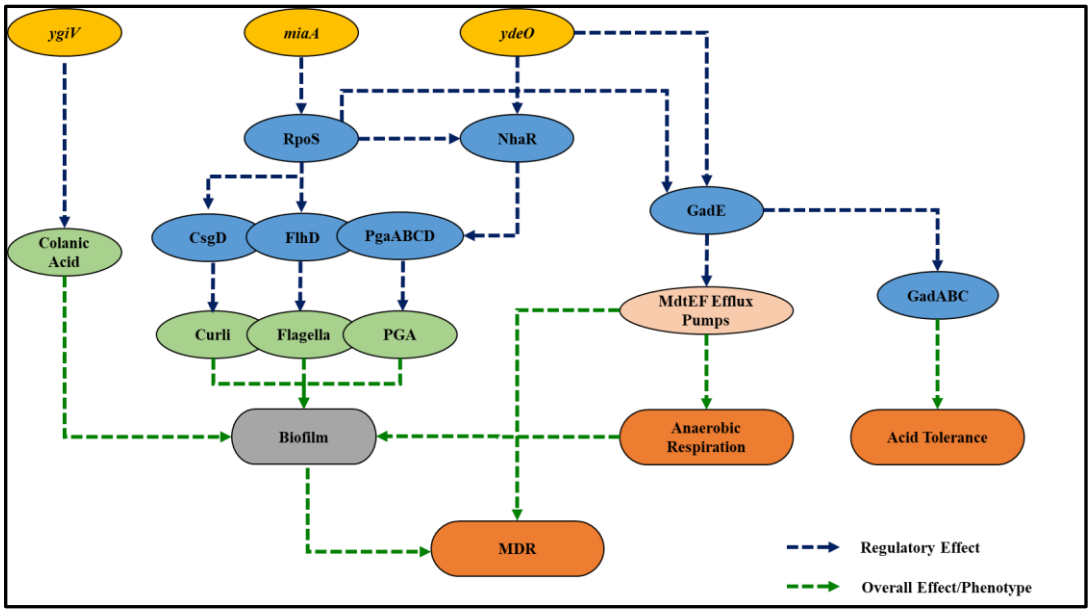


Figure 12. Regulatory network and the overall effect of the target genes *miaA*, *ydeO*, and *ygiV* on *E. coli* biofilm formation and multi-drug resistance (MDR). The three genes *miaA*, *ydeO*, and *ygiV* were identified as novel targets via predictive studies with the *in silico* platform and further validated for their roles in biofilm formation in *E. coli* clinical isolates. The regulatory network (with their representative components) for the three genes involves different components of biofilm and multi-drug resistance.

The three gene products identified by the model simulations are crucial for their overall impact on different aspects of biofilm formation and AMR: (A) The gene *miaA*, which encodes dimethylallyl diphosphate:tRNA transferase, affects the expression of RpoS, a global regulatory sigma factor, which regulates the expression of genes associated with structural components of biofilm, like curli and flagella, as well as biofilm matrix component like PGA (poly-beta-1,6-N-acetyl-D-glucosamine), via

NhaR and PgaABCD operon. (B) The gene *ydeO*, a transcription factor in *E. coli* belonging to the AraC/XylS family, also regulates the synthesis of PGA adhesin, which is another essential component of the biofilm matrix. Furthermore, it also drives the expression of genes contributing to acid tolerance (GadE), as well as RND efflux pumps MdtEF, reported to contribute to multi-drug resistance and tolerance to anaerobic conditions in *E. coli*[57]. (C) Finally, the third target, *ygiV*, another transcription regulator is directed toward the release of colonic acid, another crucial component of the biofilm matrix. The contribution of each of these genes in *E. coli* biofilm formation was further, assessed by *in vitro* assays. As represented in **Figure 13**, the knockdown of the identified target genes in clinical isolates (uropathogenic *E. coli*, UPEC) as well as the common laboratory strain (*E. coli* BW25113), resulted in a substantial reduction in biofilm formation. Further, qRT-PCR-based quantification led to the finding of their increased levels in biofilm-forming strains implicating a role in pathogenicity and resistance.

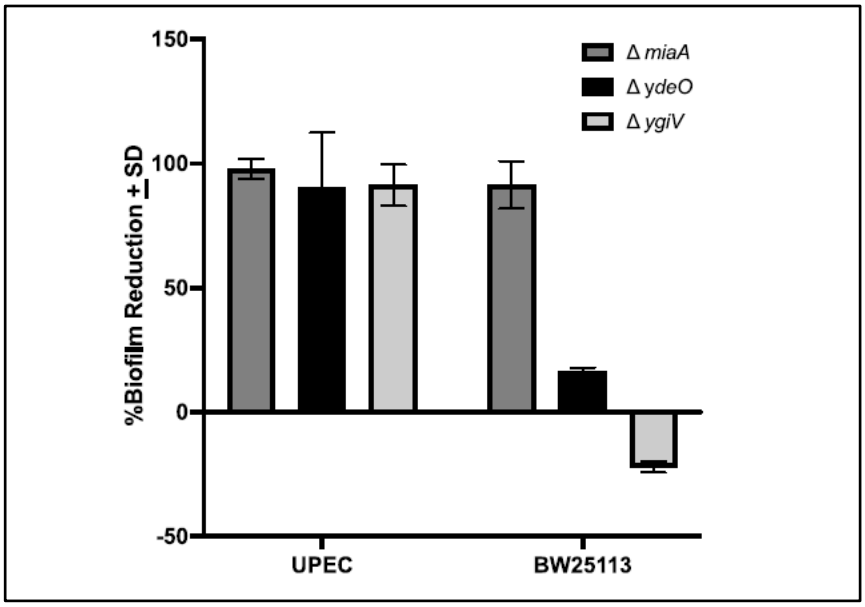


Figure 13. Inhibition of biofilm formation with specific UPEC and *E. coli* **BW25113 target KOs.** KO strains of miaA, ydeO, and ygiV, generated in UPEC and *E. coli* BW25113 (from Keio collection[58]) were assessed for biofilm formation using the CV biofilm assay. % reduction was calculated based on biofilm formation seen with the host strains, UPEC, and *E. coli* BW25113 respectively. Data presented are the mean (%) ± SD of biofilm formation as measured in the CV assay with replicates.

Thus, the *in silico* platform development led to the identification of key genes *miaA*, *ydeO*, and *ygiV* whose inhibition by small molecule inhibitors could lead to a reduction in biofilm formation and consequently aid in clearing chronic infections normally associated with biofilm-forming pathogens. Moreover, this could also indirectly improve sensitivity to standard drugs by reducing efflux-mediated resistance and recalcitrance. Findings reported in this study also offer opportunities for identifying biofilm inhibitors with applications in other multiple industries. The *in silico* framework reported here is open source and accessible for further enhancements.

D. Summary and Conclusion.

Discovery of an effective antibacterial agent requires inhibition of either one or more essential targets which causes inhibition of growth and consequently affects its survival. To escape such a deleterious action of antibiotics, the pathogen develops resistance through multiple avenues. Among the different mechanisms that bacteria employ to overcome antibiotics action, the use of efflux pumps and forming biofilms contribute substantially to the frequently acquired resistance traits. Both phenomena benefit the bacteria to avoid antimicrobials and promote antimicrobial resistance (AMR) akin to 'two peas in a pod', as they are often found to be associated together with incidents of multi-drug resistance in clinical isolates of hospital-acquired infections. The current work is an attempt to unravel the workings of these constantly evolving efflux pumps and the extensive structural changes that bacteria undergo to allow for persistence and tolerance to anti-bacterial drugs. In the case of the former, an inhibitor would interfere with the efflux pump activity thereby making them sensitive to antibiotics whereas in the case of the latter, any disruption of multi-cellular structures could lead to reduced virulence and improved susceptibility. In both cases, the pathogen is now ill-equipped to escape the efficient growth inhibitory action of an antibiotic within the host.

Therefore, in light of our current understanding and available information in the published literature, we have considered these distinct, yet significant contributors of AMR across the bacterial systems. The set research objectives and findings thereof would certainly help in a better understanding of the structural and functional characterization of different members of the RND efflux pump family and the plausible mechanisms involved in the transmission of AMR across different

bacterial systems. Furthermore, the structural and functional explorations of the OqxB's structure and its role in fluoroquinolone resistance will add significant value to the knowledge of our current understanding of the RND efflux pumps. At the same time, the *in silico* model for *E. coli* biofilm provides an overall view of the interconnecting networks and pathways contributing to biofilm formation. The model successfully identified and validated three novel target genes in *E. coli*, namely *miaA, ydeO & ygiV*, for their potential role in biofilm formation and plausibly to tolerance to harsh environments and resistance. The biofilm platform is available in the public domain as an "open-source plug-and-play" model and provides an excellent opportunity to further upgradation. This platform also offers scope for enhancements with the availability of more information, allowing researchers for an improved assessment of the role of cellular metabolites, signalling pathways, key structural and regulatory components and efflux pumps during biofilm formation and antimicrobial resistance (AMR). The link to efflux pumps reiterates the importance of this key strategy used by pathogens to better survive stressful environments. The present research would significantly aid in designing agents that could interfere with efflux pump inhibition and/or disruption of biofilms, with specific applications in different industrial sectors, including healthcare.

In summary, the output of this thesis work would certainly add value to the ongoing research on understanding and devising possible strategies to tackle AMR, contributing to the discovery and development of countermeasures for the effective treatment of multi-drug resistant infections and in improved patient care worldwide.

E. List of Publications out of PhD work

E.1. Nagakumar Bharatham, **Purnendu Bhowmik**, Maho Aoki, Ui Okada, Sreevalli Sharma, Eiki Yamashita, Anirudh P. Shanbhag, Sreenath Rajagopal, Teby Thomas, Maitrayee Sarma, Riya Narjari, Savitha Nagaraj, Vasanthi Ramachandran, Nainesh Katagihallimath, Santanu Datta & Satoshi Murakami. **Structure and function relationship of OqxB efflux pump from *Klebsiella pneumoniae***. *Nature Communications. Volume 12, Article number: 5400 (2021)*. doi: 10.1038/s41467-021-25679-0; PMID: 34518546.
Publication Date: September 13, 2021

E.2. **Purnendu Bhowmik**, Sreenath Rajagopal, Rothangamawi Victoria Hmar, Purnima Singh, Pragya Saxena, Prakruthi Amar, Teby Thomas, Rajani Ravishankar, Savitha Nagaraj, Nainesh Katagihallimath, Ramanujan Kadambi Sarangapani, Vasanthi Ramachandran, and Santanu Datta. **Validated In Silico Model for Biofilm Formation in *Escherichia coli*.** *ACS Synthetic Biology. 2022, 11, 2, 713–731.* https://doi.org/10.1021/acssynbio.1c00445; PMID: 35025506
Publication Date: January 13, 2022

E.3. **Purnendu Bhowmik**, Nagakumar Bharatham, Satoshi Murakami, Vasanthi Ramachandran, Santanu Datta. **Identification of key amino acid residues in OqxB mediated efflux of fluoroquinolones using site-directed mutagenesis**. *Research in Microbiology. Volume 174, Issue 4, May 2023, 104039.* doi: 10.1016/j.resmic.2023.104039; PMID: 36738814.
Publication Date: February 03, 2023

F. List of Conferences/Paper presentations

F.1. Oral Presentation

F.1.1 Validated *in silico* model for biofilm formation in *Escherichia coli*. Presented at "Advances in Microbial Biotechnology: Current Trends And Future Prospects". Organized by the Department of Microbiology, Biotechnology & Food Technology in Association with Microbiologists Society, India (MSI), Bangalore University. (April 28-29, 2022)

F.1.2 Structure and function relationship of OqxB efflux pump from *Klebsiella pneumoniae*. Presented at 62nd Annual International Conference of Association of Microbiologists of India (AMI) "Microbiology and Society: Current Trends and Future Prospects" (MSCTFP-2022). Organized by University of Mysore, in association with CSIR-CFTRI, DRDO-DFRL, KSTA and JSS AHER Mysuru. (September 21-23, 2022)

F.2 Conference Participation

"Antimicrobial resistance (AMR) Man Vs Microbes: Race of the Century". International symposium on Anti-microbial resistance, Biocrest 2021. Organized by School of Biotechnology at Amritha Vishwa Vidyapeetham, CHARM at University of California (San Diego), Bugworks Research Inc. and C-CAMP. (February 24-26, 2021)

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